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ANAEROBIC, NITRATE-DEPENDENT OXIDATION OF U(IV) OXIDE MINERALS BY THE CHEMOLITHOAUTOTROPHIC BACTERIUM THIOBACILLUS DENITRIFICANS

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Running title: Nitrate-dependent U(IV) oxidation by *T. denitrificans*

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Abstract

Under anaerobic conditions and at circumneutral pH, cells of the widely-distributed, obligate chemolithoautotrophic bacterium *Thiobacillus denitrificans* oxidatively dissolved synthetic and biogenic U(IV) oxides (uraninite) in nitrate-dependent fashion: U(IV) oxidation required the presence of nitrate and was strongly correlated to nitrate consumption. This is the first report of anaerobic U(IV) oxidation by an autotrophic bacterium.

In situ microbial reductive immobilization of radionuclides in aquifers and saturated soils is a remedial approach that has been the subject of considerable research effort over the past decade. The essence of this approach is that many radionuclides of concern are redox-active and are less soluble in their reduced form, and thus can be immobilized in aquifers via microbially mediated reduction under anaerobic conditions. For example, U(VI) in the form of UO_2^{2+} and its complexes is relatively water soluble, whereas the mineral uraninite (UO_2), typically formed by U(VI)-reducing bacteria (5, 10), has very low solubility. Since the earliest reports of direct microbial reduction of uranium by *Geobacter metallireducens*, *Shewanella oneidensis* (formerly *S. putrefaciens*), and *Desulfovibrio desulfuricans* (9-11), other species have also been shown to have this capability. However, recent studies have suggested that microbially mediated U(IV) oxidation could complicate efforts at long-term reductive immobilization. In 2002, two articles reported that uranium re-oxidation can occur anaerobically in the presence of nitrate, which is a common co-contaminant with uranium at U.S. Department of Energy sites (13). Finneran et al. (4) showed that nitrate-grown, but not Fe(III)-grown, cells of *Geobacter metallireducens* carried out nitrate-dependent U(IV) oxidation in anaerobic cell suspensions amended with UBr_4, a soluble form of U(IV). Senko et al. (14) observed nitrate-dependent uranium solubilization

during push-pull (*in situ*) field studies and invoked an indirect, microbial mechanism [namely, abiotic oxidation of U(IV) by intermediates of bacterial nitrate reduction, such as nitrite]. Although they invoked an indirect mechanism, Senko et al. (14) could not rule out direct microbial oxidation of U(IV) based on their data.

In this article, we report that the widely-distributed, obligate chemolithoautotrophic bacterium *Thiobacillus denitrificans*, known for its ability to couple the oxidation of various S- and Fe(II)-containing electron donors with denitrification (e.g., 8, 15), is capable of anaerobic, nitrate-dependent oxidative dissolution of synthetic and biogenic U(IV) oxides. *T. denitrificans* has relevance to certain uranium-contaminated sites, as this species (or species with >98% 16S rDNA sequence similarity) was found to account for a relatively large proportion of the bacterial community from an open-pit uranium mine (representing ~24% of the 16S rDNA clones analyzed; 16). This is the first report of anaerobic, nitrate-dependent U(IV) oxidation by an autotrophic bacterium, although the aerobic oxidation of U^{4+} (aq) by the autotrophic bacterium *Acidithiobacillus ferrooxidans* (formerly *T. ferrooxidans*) at pH 1.5 was reported by DiSpirito and Tuovinen (3).

All experiments described in this article were performed at 30°C under strictly anaerobic conditions in an anaerobic glove box (Coy Laboratory Products, Inc., Grass Lake, Mich.) with a gas composition of approximately 90% N_2 – 8% CO_2 – 2% H_2. The glass and plastic materials used to contain or manipulate the cultures were allowed to degas in the glove box for at least several days before use. *T. denitrificans* (ATCC strain 25259, obtained from the American Type Culture Collection) was cultivated in anaerobic growth medium (pH ~ 7) that included the following compounds added at the concentrations (mM) specified in parentheses: $Na_2S_2O_3 \cdot 5H_2O$ (20 mM), NH_4Cl (18.7 mM), KNO_3 (20 mM), KH_2PO_4 (14.7 mM), $NaHCO_3$ (30 mM),

$MgSO_4 \cdot 7H_2O$ (3.25 mM), $FeSO_4 \cdot 7H_2O$ (0.08 mM), $CaCl_2 \cdot 2H_2O$ (0.05 mM), and anaerobic and sterile vitamin, trace element, and selenite-tungstate solutions prepared as described by Widdel and Bak (18)(stock solutions 1, 4, 6, 7, and 8). Anaerobic techniques used in the preparation of growth medium and stock solutions are described elsewhere (1). Highly-purified water (18 MΩ resistance) obtained from a Milli-Q UV Plus system (Millipore, Bedford, MA) was used to prepare the growth medium and all other aqueous solutions described in this article. For U(IV) oxidation experiments, *T. denitrificans* cells in late exponential phase (~9 x 10^7 cells/mL) were harvested anaerobically by centrifugation in sealed polycarbonate bottles and washed once in an anaerobic resuspension buffer. The composition of the resuspension buffer was similar to that of the growth medium with the following exceptions: it contained no $Na_2S_2O_3 \cdot 5H_2O$, $CaCl_2 \cdot 2H_2O$, or KNO_3, the KH_2PO_4 concentration was reduced to 1.5 mM [to effectively preclude the formation of soluble U(IV)-phosphate complexes, based on geochemical modeling with PHREEQC (12)], and the $FeSO_4 \cdot 7H_2O$ concentration was reduced to 0.0075 mM [to preclude the possibility that iron could markedly enhance U(IV) oxidation, as has been documented in studies with *G. metallireducens* involving $FeSO_4$ concentrations of 5 to 6 mM (4)]. For cell suspension experiments, the resuspension buffer was amended with additional compounds, as described later.

Assays for U(IV) oxidation were performed in 25-mL serum bottles sealed with butyl rubber stoppers and containing either 5 or 6 mL of liquid culture, depending on the experiment. Positive controls to assess batch-specific denitrification activity of *T. denitrificans* cells were carried out in the growth medium (i.e., with thiosulfate as the electron donor) and received the same inoculum (in terms of number and concentration of cells) as cultures assayed for U(IV) oxidation. Dissolved uranium was determined according to the following steps: (i) passage of

samples through 0.2-μM syringe filters under anaerobic conditions, (ii) immediate centrifugation of the filtrate (~20,000 x *g*, 4°C, 4 min) to minimize the possibility that colloidal U(IV) could be included in the analysis (although colloidal material was never visible in the filtrates), (iii) addition of 50 (or 500) μL of the supernatant to 4950 (or 4500) μL of 0.32N HNO_3 containing thallium (10 μg/L) as an internal standard, and (iv) analysis of the resulting solution by inductively coupled plasma-mass spectrometry (ICP-MS). ICP-MS analysis, which was conducted with a quadrupole, single-collector instrument (model 4500, Agilent Technologies, Palo Alto, Cal.), attained precision within 5% and accuracy >95% using standards traceable to the National Institute of Standards and Technology. The underlying assumption of this analytical method, namely that dissolved uranium would be solely in the +VI oxidation state under the experimental conditions, was founded on geochemical modeling with PHREEQC (12) and was tested for selected samples by comparing results of ICP-MS analysis to those of kinetic phosphorescence analysis (KPA) (2), which is specific to U(VI). Nitrate and nitrite were determined by ion chromatography (model DX 500, Dionex Corporation, Sunnyvale, Cal.) with micromembrane suppression and electrochemical conductivity detection. Protein concentrations were determined with a Coomassie dye-protein binding colorimetric method (Pierce Biotechnology, Rockford, Ill.) after hydrolysis of the cells in 0.5N NaOH at 100°C for 10 min; bovine serum albumin was used as the standard.

T. denitrificans cells catalyzed oxidative dissolution of U(IV) oxide in anaerobic resuspension buffer containing ca. 3 mM nitrate and ca. 1 mmol/L synthetic U(IV) oxide (Figure 1a). To confirm that the dissolved uranium that appeared over time was truly oxidized to U(VI), two active samples collected on Day 17 were subjected to KPA. For both samples, the ICP-MS and KPA results agreed within 4%, thereby confirming oxidative dissolution. Live (without

nitrate) and sterile controls indicated that oxidative dissolution was nitrate-dependent and was catalyzed by active *T. denitrificans* cells: the increase in dissolved uranium in the live (without nitrate) and sterile controls was <3% of that observed in the active cultures (Table 1). The live controls differed from the active cultures only in that the controls contained no nitrate; the sterile controls differed from the active cultures only in that the cells were autoclaved (anaerobically) before addition to the bottles. In this experiment, U(IV) oxide was produced under strictly anaerobic conditions by adding 0.1 mmol of uranyl acetate dihydrate to 30 mL of sterile, degassed Milli-Q water, and then adding 1 mmol of sodium dithionite that had been stored in the glove box. The precipitate was washed three times with sterile, degassed Milli-Q water. The washed U(IV) oxide pellet, which had a slurry consistency, was added to serum bottles with a sterile spatula.

When the U(IV) oxide synthesis procedure was modified by allowing the slurry to dry for 5 days in an anaerobic glove box and form aggregated particles, results indicating nitrate-dependent U(IV) oxidation by *T. denitrificans* (Fig. 1b) were qualitatively similar to those depicted in Fig. 1a. However, the rate of U(IV) oxidation for the dried U(IV) oxide was <0.2% that observed for the U(IV) oxide slurry (Table 1 and Fig. 1). Furthermore, the percentage of total U(IV) that was oxidatively dissolved with the dried U(IV) oxide was two to three orders of magnitude lower than the percentage for the U(IV) slurry (Table 1). This difference cannot be attributed to batch-to-batch differences in the activity of *T. denitrificans* cells, as the total denitrification activities (the product of specific activity and amount of protein) of the positive controls for the experiments represented in Fig. 1 a and b agreed within 5% (Table 1). It is possible that the differences in U(IV) oxidation could be explained by greater mass transfer limitations or lower specific surface area for the dried U(IV) oxides. Unfortunately, reliable

physical characterization of the materials was precluded by the vulnerability of the U(IV) oxides to rapid oxidation in air and the possibility of associated changes in physical properties.

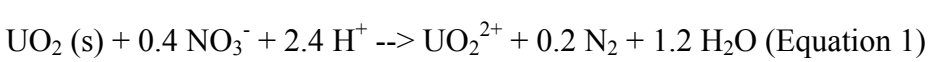
Additional studies carried out with biogenic uraninite (UO_2) also clearly indicated nitrate-dependent U(IV) oxidation by *T. denitrificans* (Figure 2a), as active cultures produced significantly more dissolved U(IV) than sterile or live controls (*t*-test, $P<0.01$). However, the background concentration of dissolved uranium was higher and the differences between active and control cultures were less pronounced as compared to the results with synthetic U(IV) oxides (Figure 2a, Table 1). Biogenic uraninite, which was graciously provided by J. Fredrickson (Pacific Northwest National Laboratory, or PNNL), was generated with H_2-oxidizing suspensions of *Shewanella putrefaciens* CN32 (6) and has been characterized by X-ray diffraction (5). In the present study, biogenic UO_2 was dispensed from a 7.5 mmol/L stock solution in anoxic water to attain a final concentration of approximately 180 μmol/L.

Nitrate and nitrite data were collected for suspensions with biogenic uraninite. Nitrite was never detected (detection limit, ca. 10 μM) during the experiment depicted in Figure 2. Thus, abiotic reaction of dissolved nitrite with uranium is unlikely to have accounted for the observed net oxidation of 39 μM U(IV), which would have required from 13 to 78 μM nitrite, depending on whether nitrite were reduced to ammonium or nitrogen monoxide, respectively. Both U(IV) oxidation and nitrate consumption slowed over time (Fig. 2), and in fact, these processes were strongly correlated over time (Fig. 3; $r^2 = 0.98$). Despite this strong linear correlation, it is clear that U(IV) could not have served as the predominant electron donor for denitrification, as the net amount of U(IV) oxidized (39 μM) is <2% of the amount that would be required to denitrify the 0.96 mM of nitrate consumed.

Hydrogen (present in the glove box atmosphere to drive the palladium, oxygen-consuming catalyst) is the only electron donor that could have accounted for nitrate consumption [note that Fe(II), present at 7.5 μM, could account for <0.2% of the observed denitrification]. An initial concentration of 1.8% H_2 in the headspace of active cultures (which is generally consistent with the H_2 monitor in the glove box) would account for reduction of ca. 1 mM nitrate to N_2, and could explain why nitrate consumption effectively ceased by Day 14 (i.e., the primary electron donor was depleted). Another possible explanation for the gradual decrease in U(IV) dissolution rate (Fig. 2a) is that oxidation somehow passivated the mineral surface, for example, by altering the surface composition. Despite the decrease in U(IV) oxidation rate, a larger portion of the total U(IV) was dissolved in the experiment with biogenic uraninite than in those with synthetic U(IV) oxides (Table 1).

Whether or not nitrate-dependent U(IV) oxidation is coupled to energy conservation in *T. denitrificans* and *G. metallireducens* cannot be determined from existing data. The overall reaction (Eq. 1) is thermodynamically favorable ($\Delta G^{o'}$ = -65 or –93 kJ/mol for crystalline or amorphous UO_2, respectively, based on data from references 7 and 17), although the reduction potential of the $UO_2^{2+}/UO_2(s)$ couple is relatively high (E_o' = 0.41 or 0.26V for crystalline or amorphous UO_2, respectively) and would thus require a relatively high-potential electron transport carrier (possibly a *c*-type cytochrome). If U(IV) oxidation were coupled to nitrate reduction to nitrite rather than denitrification, it would yield little free energy (E_o' = 0.43V for the NO_3^-/NO_2^- couple). The strong correlation between U(IV) oxidation and nitrate consumption (Fig. 3) cannot be used to definitively conclude that these processes were coupled for energy conservation, as U(IV) oxidation only accounted for <2% of nitrate consumption and ceased when nitrate was still available, which may indicate that U(IV) oxidation required ongoing

denitrification coupled to another electron donor. Notably, U(IV) oxidation also accounted for a small portion of nitrate consumption (no more than ca. 10%) during nitrate-dependent U(IV) oxidation by the heterotroph *G. metallireducens* in cell suspension experiments (4), although it is not clear what electron donor drove nitrate reduction in that system. The exact nature of the nitrate-dependence of U(IV) oxidation, for example, whether or not proteins involved in nitrate reduction have any direct role in U(IV) oxidation, remains to be determined.



$$UO_2\ (s) + 0.4\ NO_3^- + 2.4\ H^+ \rightarrow UO_2^{2+} + 0.2\ N_2 + 1.2\ H_2O \quad \text{(Equation 1)}$$

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FIGURE LEGENDS

FIG. 1. Anaerobic, oxidative dissolution of two different synthetic U(IV) oxides by washed cell suspensions of *T. denitrificans*: (A) freshly-prepared U(IV) oxide with a slurry consistency, (B) aggregated grains of U(IV) oxide that had dried under anaerobic conditions. Datum points represent the averages of duplicate suspensions.

FIG. 2. Production of dissolved uranium (A) and consumption of nitrate (B) during anaerobic, oxidative dissolution of biogenic uraninite by washed cell suspensions of *T. denitrificans*. Datum points represent the averages of triplicate suspensions and error bars represent one standard deviation (error bars are plotted but not visible in all cases).

FIG. 3. Correlation between net U(IV) oxidation and nitrate consumption during the oxidative dissolution of biogenic uraninite by washed cells of *T. denitrificans* (experiment represented in Fig. 2). Datum points represent the averages of triplicate suspensions. A linear regression fit is plotted.

TABLE 1. Summary of experiments testing nitrate-dependent U(IV) oxidation by *T. denitrificans*.

U(IV) oxide form	Net U(IV) dissolved (μM) [a]			Portion of U(IV) dissolved (%)[b]	Positive control specific denitrification activity[c]	Protein (mg)
	Active culture	Sterile control	Live control without nitrate			
Synthetic, slurry[d]	42	0.92	0.18	4	1.1	1.1
Synthetic, dried[e]	0.067	0.0027	<0	0.02	1.4	0.9
Biogenic UO_2[f]	39	15	13	22	4.5	0.7

a - Net U(IV) dissolved is defined as the final U concentration minus the initial U concentration. Values represent the averages of duplicate cultures for synthetic U(IV) oxides or of triplicate cultures for biogenic uraninite.

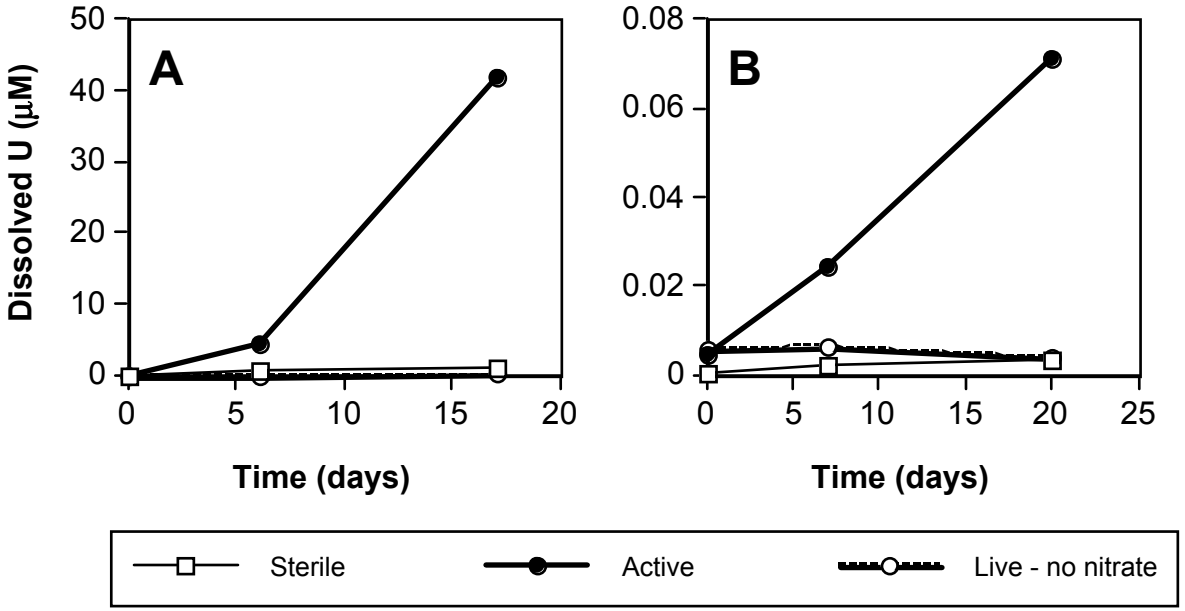
b – Approximate fraction of initial U(IV) that was dissolved in the active cultures, expressed as a percent.

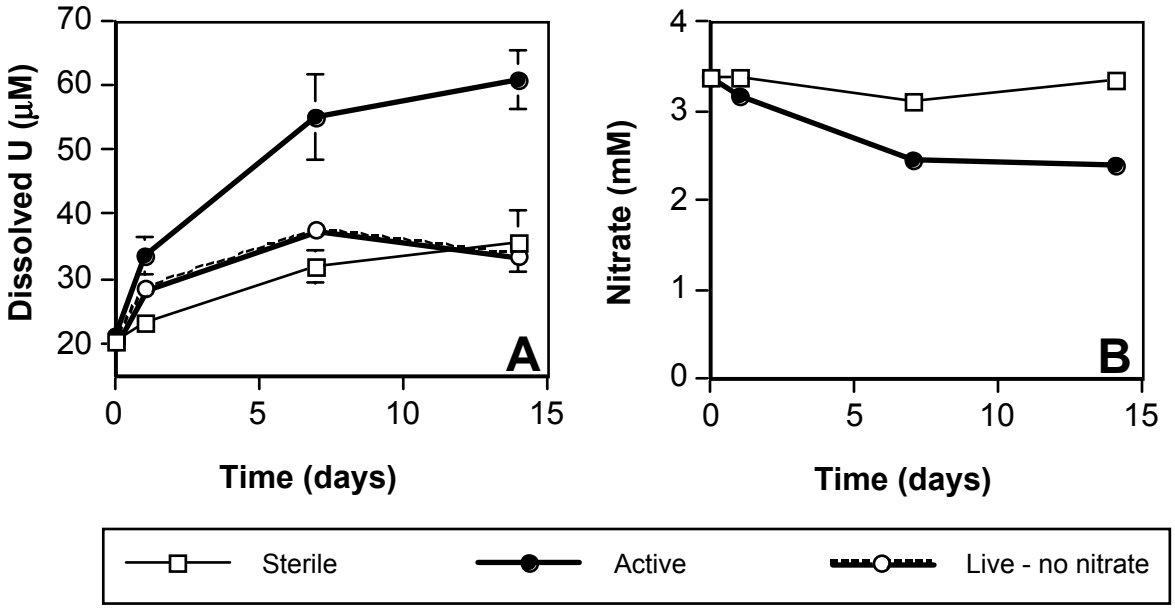
c – Units: μmol electron equivalents $\cdot$ min^{-1} $\cdot$ mg $protein^{-1}$. Positive controls contained thiosulfate as the electron donor rather than a U(IV) oxide (see text).

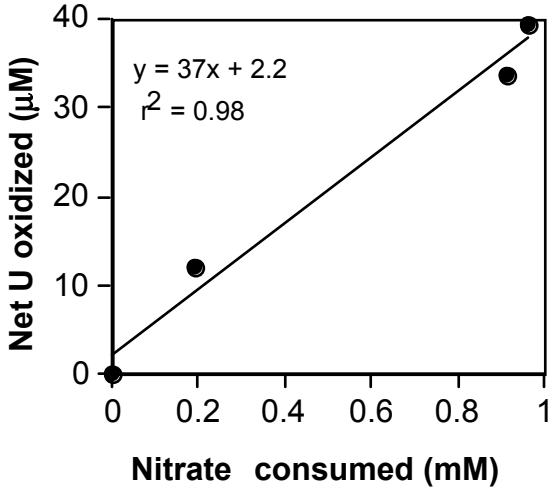
d – See Fig. 1a. Net U(IV) dissolved data are calculated for Day 17.

e – See Fig. 1b. Net U(IV) dissolved data are calculated for Day 20.

f – See Fig. 2a. Net U(IV) dissolved data are calculated for Day 14.


A
B
Dissolved U (μM)
Time (days)
Time (days)
50
40
30
20
10
0
0.08
0.06
0.04
0.02
0
0
5
10
15
20
25
Sterile
Active
Live - no nitrate


Dissolved U (μM)
70
60
50
40
30
20
A
0
5
10
15
Time (days)
Nitrate (mM)
4
3
2
1
0
B
0
5
10
15
Time (days)
Sterile
Active
Live - no nitrate


y = 37x + 2.2
r2 = 0.98
Net U oxidized (µM)
Nitrate consumed (mM)
0
10
20
30
40
0
0.2
0.4
0.6
0.8
1